Discovery of novel peptides of avian β-defensins as alternatives to control necrotic enteritis in commercial broiler chickens

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INTRODUCTION

In the world, necrotic enteritis (NE) is among the most important infectious diseases in chickens. Recently, NE has re-emerged as a significant problem as a result of restricted use of in-feed antibiotics, high-density housing conditions, and re-use of litter. Thus, there is an urgent need to develop rational, and alternative immunological management strategies not only to control, but also to prevent NE. Thus, better understanding of host-pathogen, as well as pathogen-pathogen (*Clostridium-Eimeria*) interactions in NE will be required to realize these goals. β-defensins represent important effector molecules of host innate immunity and they have been isolated from leukocytes and epithelial cells of skin, gastrointestinal, and respiratory tracts. Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are known to be potent inducers and up-regulators of defensins such as human beta defensin-2, hBD-2 (Hancock and Diamond, 2000; McDermott, 2006).

Therefore, we examined the expression profiles of AvBD transcripts in three different tissues to compare different AvBD involvement in NE in two commercial broiler chicken strains showing disparate NE disease susceptibility.

MATERIALS & METHODS

► *Animals and necrotic enteritis infection*

Two different commercial broiler lines, C (Cobb) and R (Ross), were oral infected with 1.0 × 10^4 sporulated oocysts of *E. maxima* followed with *C. perfringens* (1.0×10^9 CFU) 4 days later.

► *Tissues collection and cDNA synthesis*

Spleen, crop and intestinal jejunum tissues were collected freshly from 5 chickens per group at 2 day post *C. perfringens* infection, and pooled for total RNA extraction. Total RNA was extracted as described (Lee, et al., 2010) and cDNA was synthesized using the StrataScript first strand synthesis system (Stratagene, La Jolla, CA).

► *Quantitative real-time PCR*

Oligonucleotide primers for chicken β-defensin, pro-inflammatory cytokines, and chicken GAPDH control were designed based upon sequences available from public databases and listed in Table 1. Amplification and detection were carried out using equivalent amounts of cDNA from spleen, crop and intestine using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described (Hong, et al., 2006). The levels of individual transcripts were normalized to those of GAPDH and gene expression was analyzed by the Q-gene program (Muller, et al., 2002).

► *Statistical analysis*

Mean ± S.E. values for each group **(N = 5)** were calculated and differences between groups were analyzed by the Student's *t*-test using SPSS software (SPSS 18.0 for Windows, Chicago, IL). Statistical differences were considered significant at $p < 0.05$, $p < 0.01$, and $p < 0.0.001$.

Table 1. Sequences of the oligonucleotide primers used in quantitative RT-PCR

RNA target	Primer sequences		Size for PCR product (bp)	Accession No.
	Forward	Reverse		
GAPDH	5'-TGCTGCCCAGAACATCATCC-3'	5'-ACGGCAGGTCAGGTCAACAA-3'	142bp	K01458
Defensin-1	5'- GAAAACCCGGGACAGACG -3'	5'-AGCGAGAAGCCAGGGTGAT-3'	265bp	NM_204993
Defensin-2	5'-TCTGCAGCCATGAGGATT C -3'	5'-TAAAGCACATGCCTGGAAGAAAT -3'	259bp	NM_204992
Defensin-3	5'-ACCCAGTGCAGAATAAGAGGAG-3'	5'-AGACTGCAGAGGTGGGAAGG-3'	204bp	AY677340
Defensin-4	5'-AATCCTTTGCTTTTTCATC-3'	5'-TGCAATTATTCACACTACAACCAT -3'	201bp	NM_001001610
Defensin-5	5'-GTGACCCTCCGGGCATCT -3'	5'-ATGAACGTGAAGGGACATCAGAG-3'	253bp	NM_001001608
Defensin-6	5'-ATCCTTTACCTGCTGCTGTCTGT -3'	5'-GAGGCCATTTGGTAGTTGC -3'	250bp	NM_001001193
Defensin-7	5'-GCTGTCTGTCCTCTTTGTGGTG -3'	5'-ATTTGGTAGATGCAGGAAGGAT-3'	227bp	NM_001001194
Defensin-8	5'-TGTGGCTGTTGTGTTTTGT -3'	5'-CTGCTTAGCTGGTCTGAGG -3'	267bp	NM_001001781
Defensin-9	5'- ACCGTCAGGCATCTTCACAG -3'	5'-CCATTTGCAGCATTTCAGC -3'	241bp	NM_001001611
Defensin-10	5'-GAATTGGGGCACGCAGTC -3'	5'-CCGGAATCTTGGCACAGC -3'	229bp	NM_001001609
Defensin-11	5'-ACTGCATCCGTTCCAAAGTCTG-3'	5'-GTCCCAGCTGTTCTTCCAG-3'	168bp	NM_001001779
Defensin-12	5'- ACCTTTGTTTCGTGTTCATCTTC -3'	5'-AGGTGCTGCTGCTCTCCA -3'	230bp	NM_001001607
Defensin-13	5'- CATCGTTGTCATTCTCCTCCTC-3'	5'-GGTGGAGAACCTGCAGCAGCG-3'	163bp	NM_001001780
Defensin-14	5'-ATGGGCATATTCCTCCTG -3'	5'-CTTTGCCAGTCCATTGTAG -3'	159bp	AM402954
IL-1β	5'-TGGGCATCAAGGGCTACA-3'	5'-TCGGGTTGGTTGGTGATG-3'	244bp	NM_204524
IL-6	5'-CAAGGTGACGGAGGAGGAC-3'	5'-TGGCGAGGAGGGATTTCT-3'	254bp	NM_204628
IL-17F	5'-CTCCGATCCCTTATTCTCCTC-3'	5'-AAGCGGTTGTGGTCCTCAT-3'	292bp	NM_204460
TNFSF15	5'-CCTGAGTTATTCCAGCAACGCA-3'	5'-ATCCACCAGCTTGATGTCACTAAC-3'	292bp	NM_001024578

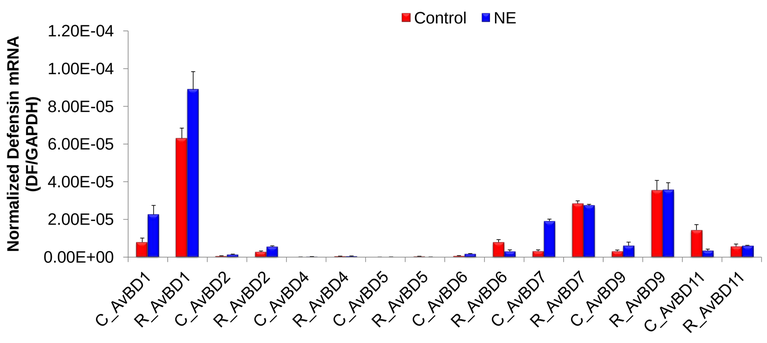

Figure 1. . The β-defensin expression profiles in the crop of two commercial broilers (Cobb vs. Ross). Chickens were non-infected or orally infected with 1.0× 10^4 oocysts of *E. maxima* on day 14 and following *C. perfringens* (C.P) infection with 1.0×10^9 CFU at 4 days later. Crops were isolated from 2 day post-C.P. infected or non-infected chickens and transcriptional levels were determined by quantitative RT-PCR. Data are expressed as normalized mRNA levels to GAPDH mRNA levels of triplicate determinations with pooled samples from five chickens.
*: P < 0.05, **: P < 0.01, ***: P < 0.001, NS: not-significant.

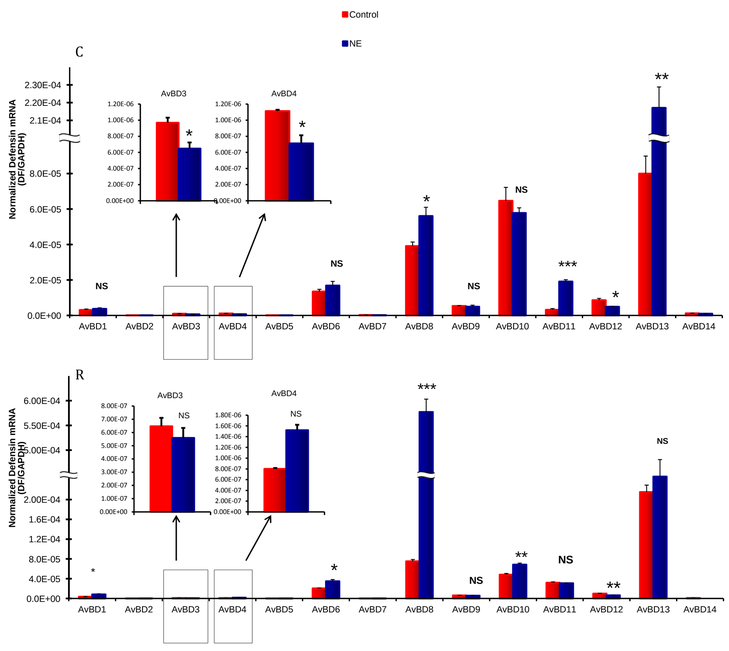

Figure 2. Chicken β-defensin expressions in the intestinal mucosal layer of two commercial broiler chickens, Cobb and Ross. Intestinal mucosal layers were isolated from the jejunum of 2 day post *C. Perfringens* infection from normal and infected chickens, and mRNA levels were determined by quantitative RT-PCR.
*: P < 0.05, **: P < 0.01, ***: P < 0.001, NS: not-significant.

REFERENCES

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RESULTS & DISCUSSION

NE was initiated in the gut by a previously established co-infection model using oral *Eimeria maxima (E. maxima)* infection followed by a *C. perfringens* challenge. Among the 14 avian β-defensin types examined, there was a tissue-specific expression of AvBD transcripts: AvBD1, AvBD7, and AvBD13 in the intestine and AvBD1 and AvBD7 in the spleen. The two different commercial broiler chicken lines showed differential gene expression patterns of AvBD transcripts following co-infection with *E. maxima* and *C. perfringens,* with Ross (R) line chickens generally showing higher expression levels than the Cobb (C) strain. Both chicken strains showed enhanced gene expression levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-17F and TNFSF15 in spleen, and TNFSF15 in intestine, whereas IL-17F was significantly increased only in the intestine of R line chickens following NE infection. Taken together, tissue- and strain-dependant expression of AvBD transcripts are induced in broiler chickens following NE infection suggesting that the local expression of these antimicrobial peptides could be important in the regulation of host innate immunity to this infection. (This project was supported by the Next-Generation BioGreen 21 No. PJ008084, RDA, Korea).

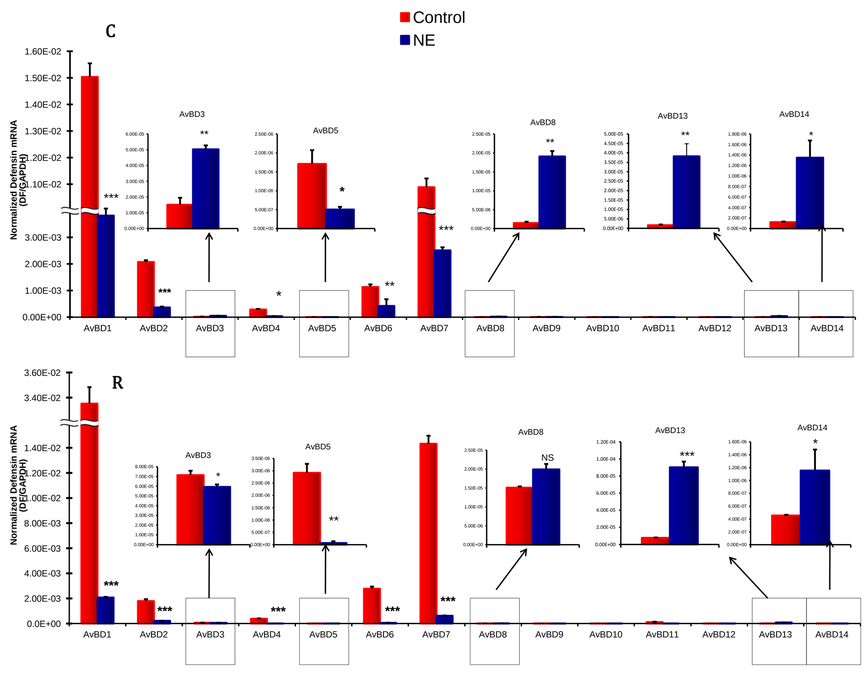

Figure 3. Chicken β-defensin expression patterns in the spleen of two commercial broiler breeds (Cobb vs. Ross). Spleens were isolated from 2 day post *C. perfringens* infection. Pooled mRNA samples from normal and *C. perfringens*-infected chickens were used in quantitative RT-PCR. Data are expressed as normalized mRNA levels to GAPDH mRNA levels of triplicate determinations with pooled samples from five chickens. To show lower level of significant β-defensin expression in one figure, small graphs were inserted in the figure. *: P < 0.05, **: P < 0.01, ***: P < 0.001, NS: not-significant.

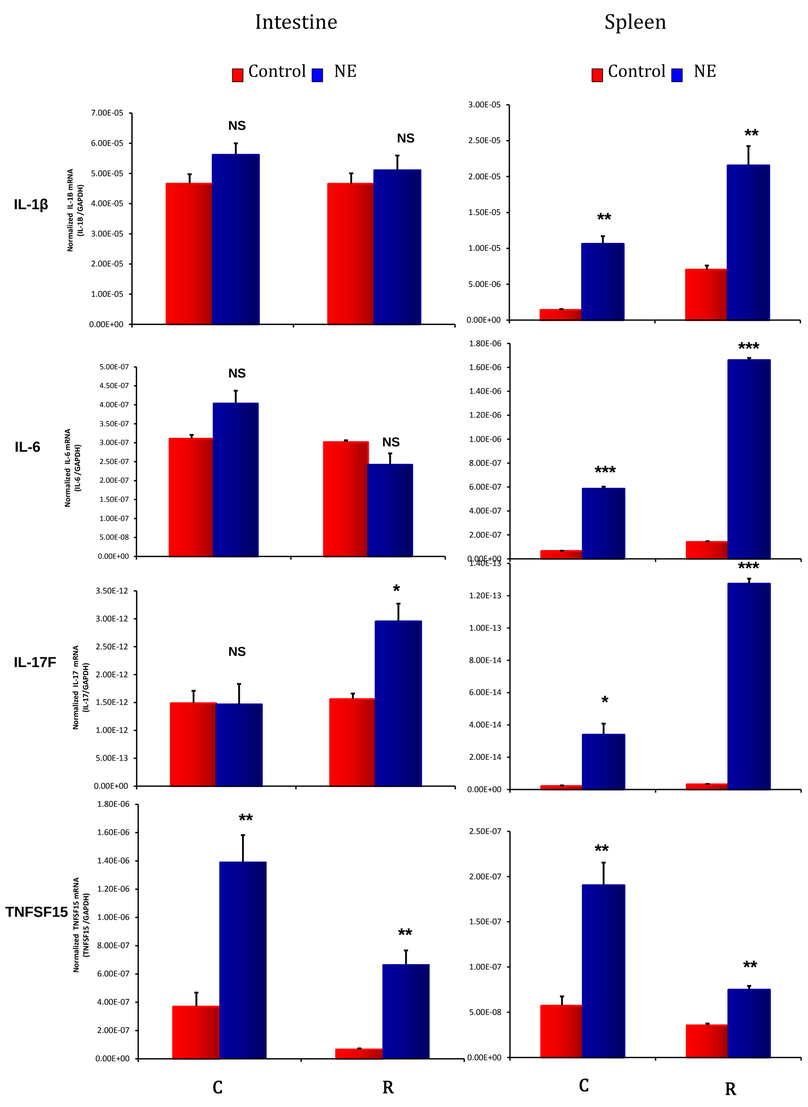

Figure 4. Pro-inflammatory cytokine mRNA levels in intestine and spleen of two commercial broiler chickens. Intestinal mucosal layer and spleen were isolated from the jejunum at the indicated time points post-infection and mRNA levels were determined by quantitative RT-PCR. Data are expressed as normalized mRNA levels to GAPDH mRNA levels of triplicate determinations with pooled samples from five chickens. *: P < 0.05, **: P < 0.01, ***: P < 0.001, NS: not-significant.